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Primer-terminus stabilization at the 3′-5′ exonuclease active site of ϕ29 DNA polymerase. Involvement of two amino acid residues highly conserved in proofreading DNA polymerases

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By site-directed mutagenesis in ϕ29 DNA polymerase, we have analyzed the functional importance of two evolutionarily conserved residues belonging to the 3′-5′ exonuclease domain of DNA-dependent DNA polymerases. In *Escherichia coli* DNA polymerase I, these residues are Thr358 and Asn420, shown by crystallographic analysis to be directly acting as single-stranded DNA (ssDNA) ligands at the 3′-5′ exonuclease active site. On the basis of these structural data, single substitution of the corresponding residues of ϕ29 DNA polymerase, Thr15 and Asn62, produced enzymes with a very reduced or altered capacity to bind ssDNA. Analysis of the residual 3′-5′ exonuclease activity of these mutant derivatives on ssDNA substrates allowed us to conclude that these two residues do not play a direct role in the catalysis of the reaction. On the other hand, analysis of the 3′-5′ exonuclease activity on either matched or mismatched primer/template structures showed a critical role of these two highly conserved residues in exonucleolysis under polymerization conditions, i.e. in the proofreading of DNA polymerization errors, an evolutionary advantage of most DNA-dependent DNA polymerases. Moreover, in contrast to the dual role in 3′-5′ exonucleolysis and strand displacement previously observed for ϕ29 DNA polymerase residues acting as metal ligands, the contribution of residues Thr15 and Asn62 appears to be restricted to the proofreading function, by stabilization of the frayed primer-terminus at the 3′-5′ exonuclease active site.

Keywords: ϕ29 DNA polymerase/3′-5′ exonuclease/site-directed mutagenesis/ssDNA binding

Introduction

Most DNA-dependent DNA polymerases are known to have an intrinsic 3′-5′ exonuclease activity, which contributes to the fidelity of DNA replication by immediate hydrolysis of polymerization errors (Brutlag and Kornberg, 1972). A detailed knowledge of the mechanism of 3′-5′ exonucleolysis has been obtained from both structural and functional high-resolution studies of the Klenow fragment of *Escherichia coli* DNA polymerase I (Pol IK), that allowed identification of the amino acid residues that bind the metal activators and DNA substrate at the 3′-5′ exonuclease active site, and analysis of their individual contribution to the reaction (Ollis *et al.*, 1985; Derbyshire *et al.*, 1988, 1991; Freemont *et al.*, 1988; Beese and Steitz, 1991). According to these studies, the major catalyst of the reaction is a pair of divalent metal ions (A and B) that are coordinated by four acidic amino acid residues, whereas a tyrosine residue plays an auxiliary role by orienting an attacking water molecule (see the review by Joyce and Steitz, 1994).

Based on amino acid sequence alignments and site-directed mutagenesis studies in ϕ29 DNA polymerase, we proposed that the 3′-5′ exonuclease active site of prokaryotic and eukaryotic DNA polymerases is evolutionarily conserved, being formed by three conserved amino acid segments (Exo I, Exo II and Exo III) that invariantly contain the five critical residues identified in Pol IK, involved in metal binding and 3′-5′ exonucleolytic catalysis (Bernad *et al.*, 1989; Blanco *et al.*, 1992; Soengas *et al.*, 1992). The validity of this proposal has been confirmed in the case of other prokaryotic and eukaryotic enzymes such as T7 (Patel *et al.*, 1991) and T4 (Reha-Krantz *et al.*, 1991; Frey *et al.*, 1993; Reha-Krantz and Nonay, 1993) DNA polymerases, *E.coli* Pol II (Ishino *et al.*, 1994), *Bacillus subtilis* Pol III (Barnes *et al.*, 1992), and cellular DNA polymerases δ (Simon *et al.*, 1991), ε (Morrison *et al.*, 1991) and γ (Foury and Vanderstraeten, 1992) from *Saccharomyces cerevisiae*. A recent steady-state analysis of mutants at each putative 3′-5′ exonuclease active site residue of ϕ29 DNA polymerase demonstrated their role in catalysis, supporting the idea that the geometry of the Pol I 3′-5′ exonuclease active site and the two-metal-ion mechanism proposed for this enzyme (Beese and Steitz, 1991) can be extrapolated to ϕ29 DNA polymerase and the other proofreading DNA polymerases (Esteban *et al.*, 1994).

Interestingly, mutational analysis of the Exo I, Exo II and Exo III motifs of ϕ29 DNA polymerase showed that the intrinsic capacity to couple strand displacement to DNA polymerization is also located in the N-terminal domain, somehow overlapping with the 3′-5′ exonuclease active site (Soengas *et al.*, 1992; Esteban *et al.*, 1994). Our current model predicts a special competition in binding the primer strand versus the displaced strand, that probably restricts the exonucleolytic action to only a mismatched primer-terminus.

In Pol IK, the physical separation of the polymerization and 3′-5′ exonuclease active sites, that is likely a general feature of proofreading DNA polymerases, constitutes an important argument for the editing decision: once a mismatch is inserted, the primer-terminus must reach the exonuclease active site by local melting of several base pairs at the 3′-terminus (Freemont *et al.*, 1988). To maintain the thermodynamic cost of this melting, a number of amino acid residues acting as single-stranded DNA (ssDNA) ligands must contribute to stabilize the primer

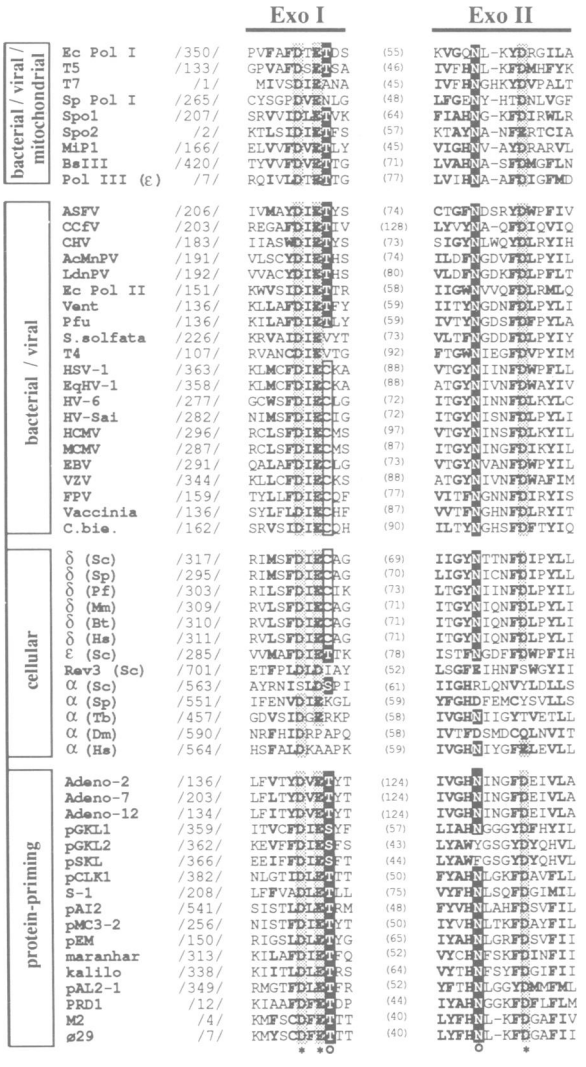

Fig. 1. Multiple alignment of amino acid sequences containing the Exo I and Exo II motifs of DNA-dependent DNA polymerases. Family A: Pol I-type DNA polymerases, including bacterial, phage and mitochondrial enzymes; family B: eukaryotic type DNA polymerases, including bacterial, viral and cellular enzymes, and those that are able to use a protein as primer. DNA polymerase nomenclature and sequence references are compiled in Braithwaite and Ito (1993), with the exception of *E.coli* DNA polymerase III ε subunit [Pol III (ε); Tomasiewicz and McHenry, 1987], African swine fever virus DNA polymerase (ASFV; Rodríguez *et al.*, 1993), DNA polymerase δ from mouse [δ (Mm); Cullmann *et al.*, 1993], DNA polymerase ε from *S.cerevisiae* [ε (Sc); Morrison *et al.*, 1991] and DNA polymerases codified by linear plasmids from *Morchella conica* (pMC3-2; Rohe *et al.*, 1991) and *Podospora anserina* (pAL2-1; Hermanns and Osiewacz, 1992). Numbers between slashes indicate the amino acid position relative to the N-terminal end of each DNA polymerase. Numbers in parentheses indicate the length of the intervening amino acid sequence. The highly conserved residues proposed to act as metal ligands at the 3′-5′ exonuclease active site are indicated by gray boxes; the corresponding residues of φ29 DNA polymerase, shown to be involved in exonucleolytic catalysis (Bernad *et al.*, 1989; Esteban *et al.*, 1994), are indicated with asterisks. The residues proposed to be homologous to Pol I Thr358 and Asn420, shown to be acting as ssDNA ligands (Beese and Steitz, 1991), are indicated in white letters over a black background; the corresponding residues of φ29 DNA polymerase, studied in this paper, are indicated with dots. Other significant amino acid similarities among the different groups are indicated in bold type. The following conservative amino acids (in one-letter notation) were considered: S and T; A and G; K, R and H; I, L, M, C, V, Y and F.

strand at the 3′-5′ exonuclease domain. By crystallographic analysis of Pol IK complexed with a tetranucleotide, a hydrophobic groove, apparently 'designed' to bind ssDNA, was shown to contain several amino acid residues directly acting as ssDNA ligands (Freemont *et al.*, 1988; Beese and Steitz, 1991). Among them, Leu361 was shown to be not essential for catalysis, but particularly important for using double-stranded DNA (dsDNA) as a substrate for 3′-5′ exonucleolysis (Derbyshire *et al.*, 1991).

Here, we describe the functional importance of two of these ssDNA ligands that, as occurs with the metal ligands forming the active site, have been preserved during evolution of the 3′-5′ exonuclease domain of proof-reading DNA polymerases.

Results and discussion

Prediction of putative 3′-5′ exonuclease active site residues

The increasing number of DNA polymerase sequences available (compiled by Braithwaite and Ito, 1993) strengthens the significance of the multiple alignments carried out in the N-terminal portion of both *E.coli* DNA polymerase I (Pol I)-type (family A) and eukaryotic type (family B) enzymes, giving further support to the hypothesis that the 3′-5′ exonuclease active site of Pol I is conserved in both prokaryotic and eukaryotic DNA polymerases (Bernad *et al.*, 1989). Thus, as shown in Figure 1, Pol I active site residues Asp355 and Glu357 (forming the Exo I motif 'DxE'), and Asp424 (forming part of the Exo II motif 'Nx_{2-3}Y/FD'), are invariant in most of the sequences aligned, with the exception of those corresponding to DNA polymerases that have no 3′-5′ exonuclease, such as cellular REV3 and DNA polymerase α. Also invariant in proofreading DNA polymerases are the residues corresponding to Tyr497 and Asp501 of Pol I that form the Exo III motif 'Yx_3D' (not shown). The main role of the carboxylic residues is proposed to be the coordination of two catalytic metal ions, whereas the Tyr residue is dedicated to orienting an attacking water molecule (Beese and Steitz, 1991).

By crystallographic analysis of Pol IK complexed with ssDNA, an additional number of amino acid residues were implicated in forming a deep crevice that holds the exonuclease substrate (Beese and Steitz, 1991). Among them, Thr358 (next to the Exo I motif) was shown to bury the 3′-OH group of the ssDNA substrate by the formation of a H-bond through its backbone amide, and Asn420 (forming part of the Exo II motif) is H-bonded to the 4′ oxygen of the penultimate nucleotide through its δ-amino group. According to the alignment shown in Figure 1, an equivalent threonine is present in seven out of nine sequences of Pol I type (family A), in three out of four sequences of bacterial DNA polymerases corresponding to the eukaryotic type (family B), and in 14 out of 17 sequences of the protein-priming group, also belonging to family B. In the latter group, three DNA polymerases from linear plasmids have the Thr changed into a conservative Ser residue. On the other hand, from a total of 17 viral DNA polymerases belonging to the eukaryotic type (family B), only five have an equivalent Thr residue. Among the cellular enzymes, only DNA polymerase ε has an equivalent Thr residue, whereas DNA

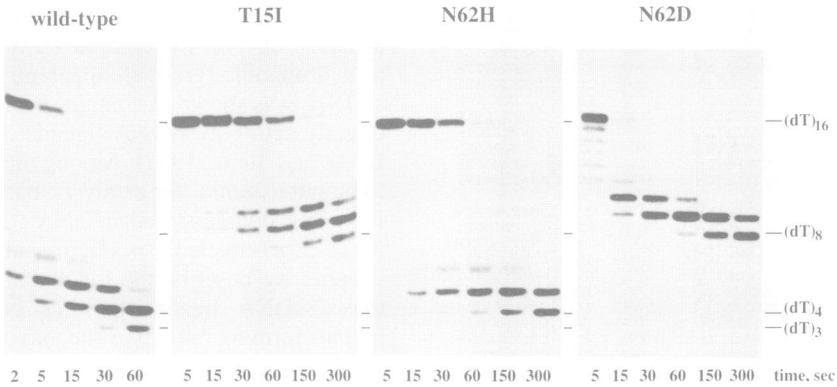

Fig. 2. 3′-5′ Exonucleolytic activity of point mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase, using oligo(dT)$_{16}$ as substrate. The assay was carried out in the conditions described in Materials and methods, in the presence of 5 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 25°C, degradation of the labeled DNA was analyzed by electrophoresis in 8 M urea–20% polyacrylamide gels and autoradiography. Total degradation was calculated as indicated in Materials and methods. Mean activity values relative to the wild-type are shown in Table I. The position of different degradation intermediates of the oligo(dT)$_{16}$ substrate is indicated. Mutations are indicated by the original amino acid (in single-letter notation), its position and the replacing amino acid: i.e. T15I = Thr15 to Ile.

polymerase δ from different sources has a Cys residue, as do most of the eukaryotic viral sequences. As occurred with some of the carboxylate residues of the Exo motifs, an equivalent Thr is absent in REV3 and DNA polymerase α. Figure 1 also shows that the Asn residue of the Exo II motif is invariant in the prokaryotic type (family A) enzymes and in the group of bacterial/viral eukaryotic type (family B) DNA polymerases. In the rest of the DNA polymerases belonging to family B, the Asn residue is invariant in 9 out of 13 cellular sequences, and in 15 out of 17 sequences of protein-priming DNA polymerases.

Therefore, the residues corresponding to Thr358 and Asn420 of Pol I appear to be evolutionarily conserved, and they can be predicted to form part of the 3′-5′ exonuclease active site of proofreading DNA polymerases.

3′-5′ Exonuclease activity of ϕ29 DNA polymerase mutants in residues Thr15 and Asn62

The functional importance of the putative active site residues described above was studied by mutagenesis of the homologous residues (Thr15 and Asn62) of ϕ29 DNA polymerase, a model enzyme for eukaryotic type DNA polymerases (see Blanco and Salas, 1995). According to Pol I data, the interaction of the Asn residue with ssDNA is mediated by the δ-amino group. Therefore, ϕ29 DNA polymerase residue Asn62 was changed either to His (N62H), to maintain a positively charged group, or to Asp (N62D), to introduce a negatively charged amino acid. In the case of the Thr residue, in which the interaction is expected to be through the backbone amide, a single change to Ile (T15I) was selected among the most infrequent substitutions observed in the alignment shown in Figure 1. Taking into account secondary structure predictions (Chou and Fasman, 1978; Garnier *et al.*, 1978) and general suggestions for conservative substitutions (Bordo and Argos, 1991), the mutation Thr15 to Ile maintains the overall structure in that region of the polypeptide. These mutant ϕ29 DNA polymerase derivatives were overproduced and purified as described in Materials and methods.

In order to measure the capacity of the mutant polymerases to degrade a ssDNA substrate, a 16mer oligo(dT) was used as substrate for 3′-5′ exonucleolysis (see Materials and methods). This homopolymer was selected to avoid the formation of any secondary structure that could complicate the interpretation of the results. As shown in Figure 2, and as had been previously reported for heteropolymeric substrates (Garmendia *et al.*, 1992), the wild-type ϕ29 DNA polymerase is able to degrade the oligo(dT)$_{16}$ in a processive way (without dissociation) until its length is reduced to a 5mer, being distributive from this position. Although the N62H mutant polymerase showed a similar pattern of degradation, its 3′-5′ exonuclease activity was estimated to be 12% that of the wild-type enzyme (see Table I). On the other hand, whereas the relative 3′-5′ exonuclease activity of mutant polymerases T15I and N62D was estimated to be 5 and 18%, respectively, the pattern of degradation was significantly different from that of the wild-type ϕ29 DNA polymerase: both mutant polymerases stopped preferentially in the 8/9mer position (see Figure 2), and only in the case of mutant T15I could the pattern of degradation reach the 5mer position when longer reaction times were used (data not shown).

The size limit for processive degradation observed with the wild-type ϕ29 DNA polymerase, and the differences observed with these two mutant derivatives, suggest differences in the stability of the DNA polymerase–oligo(dT) complexes related to the size of the DNA substrate. These differences could be established by the potential to interact with portions of the enzyme other than those corresponding to the N-terminal domain, which contains the 3′-5′ exonuclease active site. With short substrates, the enzyme–DNA interaction could be mainly dependent on specific interactions through residues located close to the active site and, therefore, degradation of those substrates could be more sensitive to mutations in these residues, which might be the case for mutants T15I and N62D. To test this hypothesis, a 4mer oligonucleotide was used as substrate for the exonucleolytic reaction (see Materials and methods). Mutant N62H was able to degrade the 4mer substrate, although with a relative catalytic efficiency of 10% that of the wild-type enzyme; the activity of mutant T15I was reduced to 2% and that of N62D to 0.7% (Figure

Table I. Enzymatic activities of wild-type and mutant derivatives at residues Thr15 and Asn62 of ϕ29 DNA polymerase

Activity assay	Substrate(s)	ϕ29 DNA polymerase			
		wt	T15I	N62H	N62D
3′-5′ exonuclease	oligo $(dT)_{16}$	100	5	12	18
	4mer	100	2	10	0.7
	template/primer	100	9	66	4
Pol/Exo balance	template/primer, dNTP	200	20	200	2
Filling-in					
DNA labeling	template/primer, dATP	100	68	75	95
dAMP release	template/primer	100	9	70	1
Misincorporation	template/primer, dATP	1	4	1	8
DNA synthesis (strand displacement)	primed M13 DNA, dNTP	100	90	74	88
	ϕ29 DNA, TP, dNTP	100	56	61	54

The different activity assays carried out with the indicated substrates are described in Materials and methods. Numbers indicate the percentage of the activity obtained with the wild-type enzyme. In the polymerase/exonuclease coupled assay, the dNTP concentration (in nM) required to efficiently elongate the 15mer primer until the 20mer position is indicated as Pol/Exo balance.

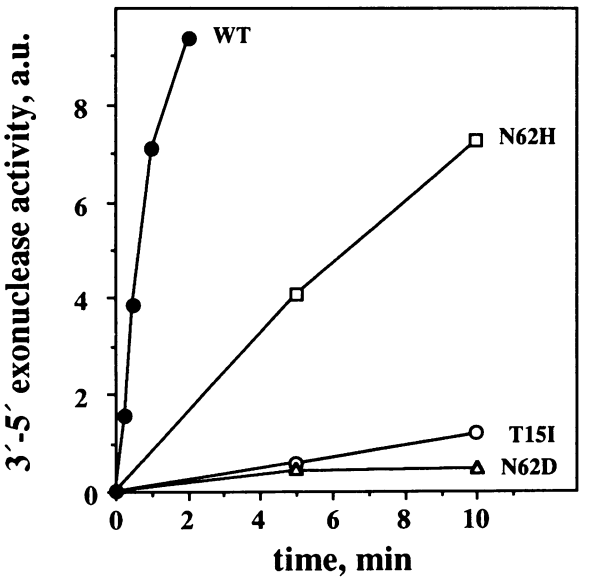

Fig. 3. 3′-5′ Exonucleolytic activity of point mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase, using a 4mer substrate. The assay was carried out in the conditions described in Materials and methods, in the presence of 25 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 25°C, degradation of the labeled DNA was analyzed by electrophoresis in 8 M urea–20% polyacrylamide gels and autoradiography. Exonucleolytic degradation, detected by the appearance of products shorter than the 4mer substrate, was quantitated by densitometry of the autoradiographs and expressed in arbitrary units (a.u.). Mean activity values relative to the wild-type are shown in Table I.

3). As can be calculated from Table I, the relative catalytic efficiency of mutant N62D fell drastically (26-fold) when the 4mer was used instead of oligo$(dT)_{16}$, whereas no significant changes were obtained in the case of mutants T15I and N62H.

The differences in processivity observed with mutants T15I and N62D, together with the proposed role of the corresponding residues of Pol I, suggest that the two mutated residues (Thr15 and Asn62) of ϕ29 DNA polymerase are involved in ssDNA binding at the 3′-5′ exonuclease active site.

Mutations at ϕ29 DNA polymerase residues Thr15 and Asn62 affect ssDNA binding

The highly conserved amino acid residues studied in this work are proposed to be the functional counterparts of Pol I residues Thr358 and Asn420, shown to be involved in binding the ssDNA which has to be degraded (Freemont *et al.*, 1988). To evaluate whether the mutations introduced in the Thr15 and Asn62 residues of ϕ29 DNA polymerase are affecting ssDNA binding, we performed a gel-retardation assay (see Materials and methods) using oligo$(dT)_{16}$ as substrate. As shown in Figure 4, the wild-type ϕ29 DNA polymerase was able to produce a single retardation band that, according to the Klenow model studies, is interpreted to be an enzyme–DNA complex in which the 3′-terminus would be located at the 3′-5′ exonuclease active site. The presence of this band absolutely depends on the absence of divalent metal ions; otherwise, exonucleolytic degradation of the ssDNA substrate is produced. As also shown in Figure 4, ϕ29 DNA polymerase mutant N62H was able to bind efficiently the ssDNA substrate; in fact, in the conditions used, the binding efficiency of this mutant was even better than that of the wild-type ϕ29 DNA polymerase. In good agreement with Pol I structural data, the wild-type-like phenotype of mutant N62H supports the proposal that the interaction of the Asn62 residue with DNA is mediated by a positively charged group. In this case, the reduced efficiency of exonucleolysis on this substrate (12%) could be due to slight differences in the orientation of the 3′-end of the substrate with respect to the active site, which could affect catalysis. On the other hand, mutant T15I was unable to bind the oligo(dT) substrate. Therefore, an unstable ssDNA binding could account for the low catalytic efficiency (5%) of ϕ29 DNA polymerase mutant T15I. Interestingly, although mutant N62D was able to bind efficiently the oligo(dT) molecule, the different mobility of the complex formed reflects an abnormal binding that could be due to a strong change in the orientation/stabilization of the 3′-end of the substrate (Figure 4). This alteration could be induced by the loss of a specific contact with DNA and/or by the introduction of a negative charge in close proximity to the ssDNA binding region. The altered

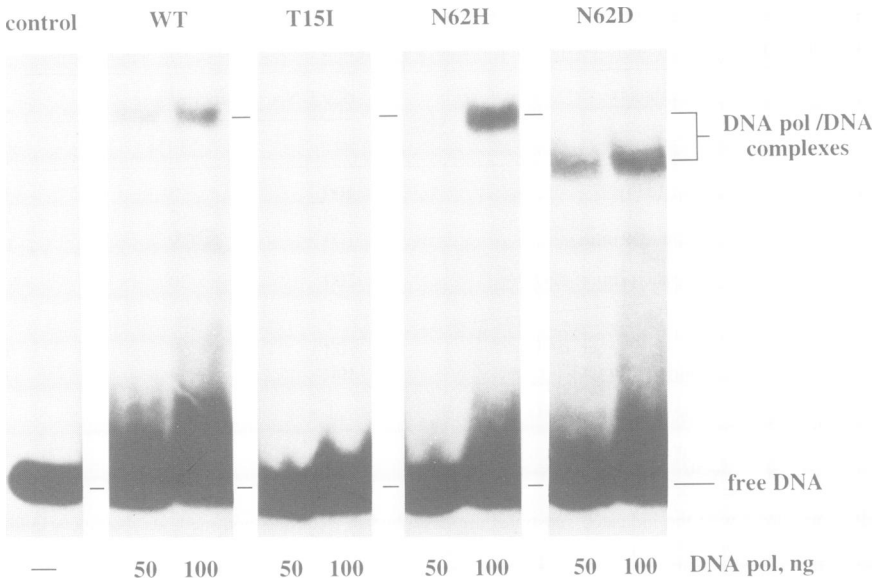

Fig. 4. Gel retardation of ssDNA by wild-type or mutant ϕ29 DNA polymerases. The assay was carried out as described in Materials and methods, using 5′-labeled oligo(dT)$_{16}$ as substrate, in the presence of the indicated amount of either wild-type or mutant ϕ29 DNA polymerase. After gel electrophoresis, the bands corresponding to free DNA and to DNA polymerase–DNA complexes, were detected by autoradiography.

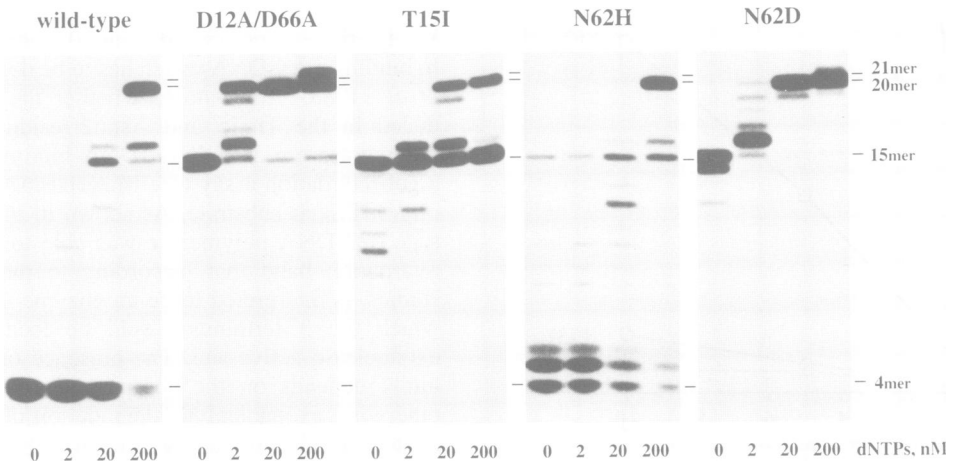

Fig. 5. DNA polymerase/exonuclease coupled assay. The assay was carried out as described in Materials and methods, using ^{32}P-labeled hybrid molecule sp1/sp1c+6 as primer/template DNA, 20 ng of either wild-type or mutant ϕ29 DNA polymerase, and the indicated concentration of the four dNTPs. After incubation for 5 min at 30°C, samples were analyzed by 8 M urea–20% PAGE and autoradiography. Polymerization or 3′-5′ exonuclease activity are detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labeled sp1 primer.

mobility obtained with mutant N62D, which could explain its reduced 3′-5′ exonuclease activity (18%), was confirmed with a 15mer single-stranded oligonucleotide (sp1) of heterogeneous sequence (data not shown).

Because the retardation assay is carried out in the absence of metal ions to avoid degradation of the oligo-(dT)$_{16}$ substrate, it is not possible to estimate how metal contributes to (or modulates) the binding of the substrate at the 3′-5′ exonuclease active site of these mutants. In this sense, it is worth noting that single and double substitutions of the active site residues involved in metal binding, belonging to Exo I, Exo II and Exo III motifs, did not affect the capacity to bind ssDNA in similar gel-retardation assays (our unpublished results). Therefore, in the absence of metal, conditions in which the binding of ssDNA directly depends on particular residues of the enzyme, amino acid residues Thr15 and Asn62 of ϕ29 DNA polymerase are important for a proper and/or stable binding of ssDNA at the 3′-5′ exonuclease active site.

ϕ29 DNA polymerase residues Thr15 and Asn62 are critical for exonucleolysis of the primer-terminus

In enzymatic terms, the optimal substrate for the 3′-5′ exonuclease activity of DNA polymerases is a molecule of ssDNA. Structural studies of Klenow fragment complexed with ssDNA provided the basis for this preference: the active site is reached through a channel, present at the N-terminal domain, that has adequate dimensions to hold 3–4 nucleotides of ssDNA (Freemont *et al.*, 1988; Beese and Steitz, 1991). However, the physiological substrate of the 3′-5′ exonuclease activity is a mismatched primer-terminus (mostly dsDNA) and, therefore, additional melting of the template/primer structure is necessary to fill the

channel and allow the mismatch to be positioned at the active site. In fact, and when melting is thermodynamically favored (high temperature, low ionic strength), a matched primer-terminus can be progressively degraded by the 3′-5′ exonuclease activity. The binding strength required to position the primer strand at the exonuclease active site must be enough to compete its stabilization at the polymerase active site, contributed by the enzyme itself and by Watson and Crick pairing with the template strand. Therefore, the importance of residues involved in ssDNA binding at the 3′-5′ exonuclease domain is expected to be more evident when the exonuclease activity is assayed on a template/primer structure. In agreement with this idea, while the ϕ29 DNA polymerase mutant N62D had its 3′-5′ exonuclease activity on ssDNA reduced 5- to 6-fold when compared with the wild-type enzyme, its activity on a template/primer was reduced 25-fold (see Table I). Therefore, the option to bind the primer at the polymerization active site aggravates the defect of 'proper stabilization' displayed by mutant N62D. On the contrary, the relative efficiency of mutants T15I and N62H on the template/primer molecule was higher (2- and 5.5-fold, respectively) than that observed on oligo(dT)$_{16}$. This partial restoration of the exonuclease activity could be explained if the binding of a template/primer molecule, as a more physiological substrate, helps to bind and/or orient the 3′-terminus at these two mutant exonuclease active sites.

The importance of ϕ29 DNA polymerase residues Thr15 and Asn62 for the degradation of the primer strand was also analyzed under polymerization conditions. As described in Materials and methods, the functional coupling between synthesis and degradation on a template/primer molecule can be evaluated as a function of the dNTP concentration. In the absence of nucleotides, the only products that can be detected are those produced by exonucleolytic digestion of the primer strand. In these conditions, the different patterns and extent of degradation obtained reflect the level of 3′-5′ exonuclease activity of the different mutants with respect to the wild-type and the exonuclease-deficient ϕ29 DNA polymerase mutant D12A/D66A (see Figure 5). On the other hand, by adding increasing amounts of dNTPs, exonucleolysis is progressively competed, favoring polymerization. As shown in Figure 5, whereas mutant N62H had the same dNTP requirement (200 nM) as the wild-type enzyme for a net polymerization balance, mutants T15I and N62D required a dNTP concentration ~10- and 100-fold lower, respectively. Interestingly, the dNTP concentration required by mutant N62D was roughly similar to that of ϕ29 DNA polymerase mutant D12A/D66A, which lacks two catalytic residues of the 3′-5′ exonuclease active site.

Similarly, the amount of dNMPs released by mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase during filling in of DNA ends (see Materials and methods) was shown to be reduced either 11-, 1.4- or 100-fold for mutant polymerases T15I, N62H and N62D, respectively (see Table I).

All these results allow us to conclude that, as expected, residues Thr15 and Asn62 are not directly involved in the polymerization activity of ϕ29 DNA polymerase; on the contrary, a critical role in primer-terminus stabilization at the 3′-5′ exonuclease active site can be deduced from the favored polymerization observed with mutants at these two residues.

Mutations at ϕ29 DNA polymerase residues Thr15 and Asn62 decrease the fidelity of DNA replication

As we have pointed out above, the capacity of mutants T15I and N62D to stabilize a 3′-end of ssDNA at the 3′-5′ exonuclease site is very affected, in particular when it represents the 3′-terminus of a primer strand. Therefore, it was interesting to analyze whether these mutants were able to eliminate a mismatched primer-terminus (see Materials and methods), a physiological situation in which a single base is mispaired and, therefore, the stabilization of the primer strand at the polymerization active site is expected to be reduced. Accordingly, mutant N62H showed a clear preference, as does the wild-type enzyme (Garmendia *et al.*, 1992), to start and continue degradation of the primer strand that was initially mismatched at its 3′-terminus (Figure 6). Similar behavior was observed with the ϕ29 DNA polymerase derivative T15I, although the exonuclease active site of this mutant was shown to be reduced in its capacity to stabilize and degrade ssDNA. In each case, the efficient degradation of the primer is probably facilitated by a thermodynamically favored melting induced by the terminal mismatch. Interestingly, in the case of mutant N62D, all the mismatched primer-termini were excised, but no further degradation of the primer strand could be detected. The selective and efficient degradation of the mismatch by this mutant indicates that its defective binding at the 3′-5′ exonuclease active site is likely compensated for not only by a thermodynamically favored melting of the template/primer structure, but also by the reduced affinity of a mismatched primer-terminus at the polymerization active site. Once the mismatch has been removed, the properly paired primer-terminus is stably bound at the polymerization active site, and the defective ssDNA binding of mutant N62D would prevent further degradation.

Once the capacity to excise a mismatched primer-terminus by the above mutants was demonstrated, the next question was whether these mutants were able to produce the insertion and stable incorporation (elongation) of mismatched nucleotides during polymerization. To address this question, a misincorporation assay was carried out (described in Materials and methods) in which the insertion of dAMP onto non-complementary positions was evaluated. As shown in Figure 7, the wild-type DNA polymerase did not produce stable misincorporation, with dAMP insertion occurring only at complementary positions 16 and 17. Also in the case of mutant N62H, dAMP was not misincorporated at non-complementary positions; the higher intensities of the bands corresponding to positions 16 and 17 are probably due to the slightly reduced (66%) exonuclease activity displayed by this mutant on template/primer molecules. In the case of mutants T15I and N62D, the appearance of label at position 19 indicates that misincorporation of dAMP and further elongation of the mismatched primer-terminus had occurred even at the lowest dATP concentration (1 μM). The faint labeling at position 18 indicates that the mispair inserted at this position can be easily elongated, suggesting that nucleotide misinsertion is the rate-limiting step. Quantification of the misincorporation produced in each case (Table I)

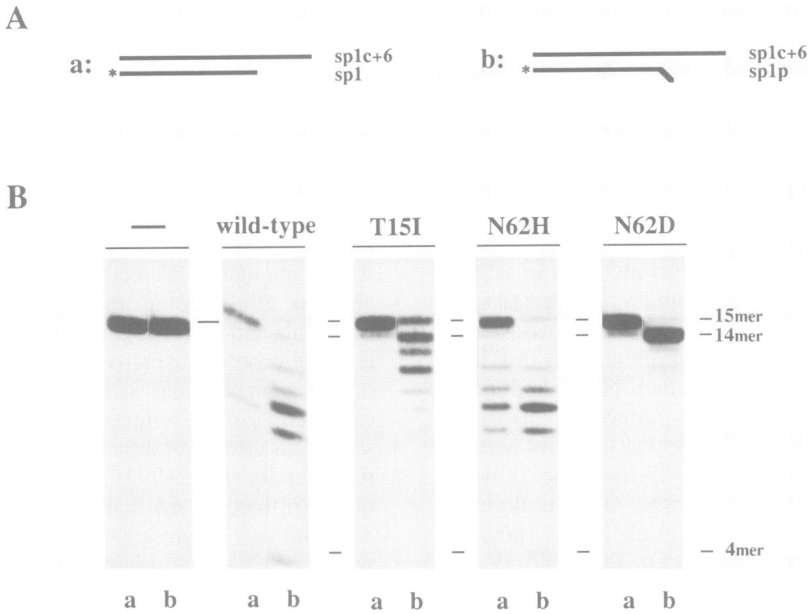

Fig. 6. Proofreading ability of site-directed mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase. (**A**) The substrates used in this assay were (a) sp1/sp1c+6 (matched) and (b) sp1p/sp1c+6 (mismatched). (**B**) The exonuclease activity assay was carried out as described in Materials and methods, using 0.15 ng of each substrate described in (A) and 20 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation at 30°C for either 2 min (wild-type and N62H) or 5 min (T15I and N62D), degradation of the 15mer primer (sp1 or sp1p) was analyzed by 8 M urea–20% PAGE and autoradiography.

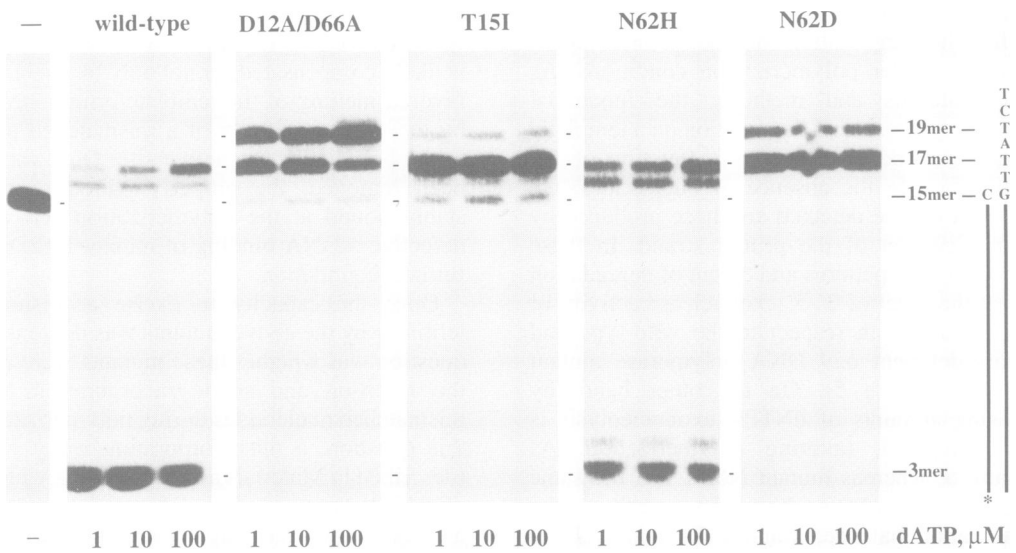

Fig. 7. Misincorporation during DNA replication by site-directed mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase. The assay was carried out in the conditions described in Materials and methods, using 0.5 ng of ^{32}P-labeled hybrid sp1/sp1c+6 (whose sequence is partially shown at the right), 20 ng of either wild-type or mutant ϕ29 DNA polymerase, and the indicated concentration of dATP. After incubation for 5 min at 30°C, the reaction products were analyzed by 8 M urea–20% PAGE, followed by autoradiography. The position corresponding to the unextended primer (15mer), and either to extended (17mer and 19mer) or degraded (3mer) products, is indicated. Relative misincorporation, calculated as the ratio 18mer + 19mer/17mer + 18mer + 19mer, is shown in Table I.

indicated that, in agreement with a reduction of the exo/pol ratio, the fidelity of DNA synthesis was reduced 4- and 8-fold in mutants T15I and N62D, respectively. In these '*in vitro*' conditions, the ϕ29 DNA polymerase double mutant D12A/D66A showed a 23-fold reduction in fidelity with respect to the wild-type enzyme. Therefore, and in spite of the strong selectivity against mismatches shown by mutant N62D (see Figure 6), the presence of the next correct nucleotide, together with the altered ssDNA binding capacity of this mutant, decrease its proofreading efficiency by favoring extension of polymerization errors.

All these results indicate that substitution of ϕ29 DNA polymerase residues Thr15 and Asn62 has significant consequences for the fidelity of DNA replication by this enzyme: in spite of the fact that mutants T15I and N62D

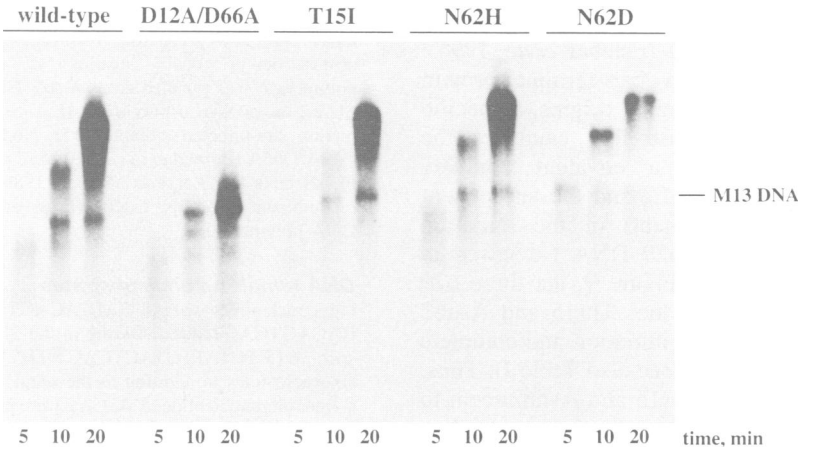

Fig. 8. Strand displacement coupled to M13 DNA replication by site-directed mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase. Replication of primed M13 DNA was carried out as described in Materials and methods using 100 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 30°C, relative activity values were calculated from dNTP incorporation (see Table I), and the length of the synthesized DNA was analyzed by alkaline 0.7% agarose gel electrophoresis and autoradiography. The position of full-length M13 DNA is shown at the right.

are able to excise a mismatched primer-terminus, their defect in the stabilization of ssDNA at the 3′-5′ exonuclease active site leads to an increase in the stable incorporation of wrong nucleotides, thus decreasing the fidelity of DNA synthesis.

Mutations at ϕ29 DNA polymerase residues T15 and N62 can carry out DNA elongation coupled to strand displacement

Strand displacement coupled to DNA synthesis is one of the most peculiar functions of ϕ29 DNA polymerase, that allows it to carry out the complete replication of the linear ϕ29 DNA genome (19 285 bp) in the absence of accessory proteins or helicases (Blanco *et al.*, 1989). It had been previously shown that all the mutations introduced in the catalytic residues corresponding to the Exo I, Exo II and Exo III motifs of ϕ29 DNA polymerase, in addition to inactivating the 3′-5′ exonuclease activity, severely affected the rate of DNA synthesis coupled to strand displacement (Soengas *et al.*, 1992; Esteban *et al.*, 1994). Taking into account these results and the fact that none of the mutations carried out in the C-terminal portion of ϕ29 DNA polymerase specifically affected strand-displacement synthesis, it was proposed that this activity would reside in the N-terminal domain, probably overlapping with the 3′-5′ exonuclease active site (Soengas *et al.*, 1992; Esteban *et al.*, 1994).

As a plausible model for strand displacement, it was speculated that the enzyme could make an alternative use of the ssDNA binding groove present in the N-terminal domain, either to bind the 3′-5′ exonuclease substrate, or to stabilize the interaction between the polymerase molecule and the DNA strand to be displaced. To test this model, ϕ29 DNA polymerase mutants at residues Thr15 and Asn62, shown here to be involved in ssDNA binding at the 3′-5′ exonuclease active site, were assayed for catalysis of DNA polymerization coupled to strand displacement using two different replication assays: primed M13 DNA replication and ϕ29 DNA replication.

In the primed M13 DNA replication assay (see Materials and methods), ϕ29 DNA polymerase starts polymerization

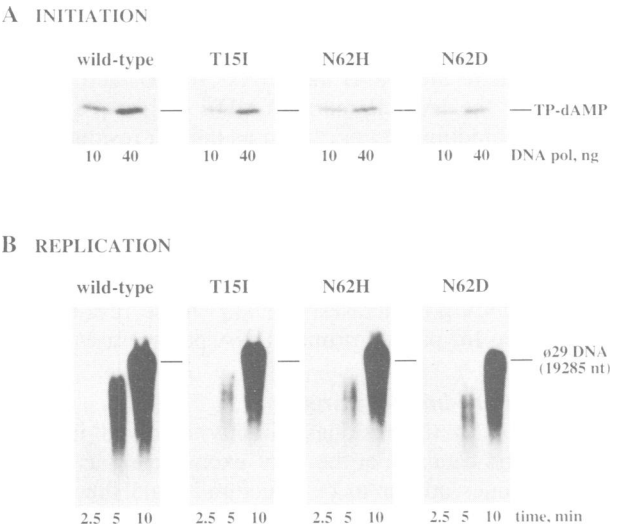

Fig. 9. ϕ29 DNA-TP replication by site-directed mutants in residues Thr15 and Asn62 of ϕ29 DNA polymerase. (**A**) TP-primed initiation activity. The initiation assay was carried out as described in Materials and methods, in the presence of the indicated amounts of either wild-type or mutant ϕ29 DNA polymerase. After incubation for 5 min at 30°C, the samples were analyzed by SDS–PAGE and autoradiography. The electrophoretic mobility of the TP–dAMP initiation complex is indicated. (**B**) Replication of ϕ29 DNA-TP. The assay was carried out as described in Materials and methods, in the presence of 10 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 30°C, relative activity values were calculated (see Table I), and the length of the synthesized DNA was analyzed by alkaline agarose gel electrophoresis. The migration position of unit-length ϕ29 DNA is indicated.

from the 3′-OH group of a short DNA primer, and once the 5′ terminus of this primer is reached, strand displacement is required for ongoing polymerization. As shown in Figure 8, the size of the replication products obtained with mutants T15I, N62H and N62D (several-fold the length of the M13 DNA template) reveals that these mutants are not affected in polymerization coupled to strand displacement. As a comparison, Figure 8 shows

the behavior of mutant D12A/D66A, strongly affected in strand displacement DNA synthesis (Esteban *et al.*, 1994).

The replication of ϕ29 DNA involves terminal protein (TP)-primed initiation at both terminal origins, a specific activity of ϕ29 DNA polymerase that catalyzes the template-directed formation of a covalent complex between the viral TP and 5′-dAMP, and the subsequent elongation (via strand displacement) of the initiation complex to produce full-length ϕ29 DNA (reviewed in Salas, 1991). As can be seen in Figure 9, the three ϕ29 DNA polymerase mutants at residues Thr15 and Asn62 were able to carry out TP-primed initiation and complete replication of the viral ϕ29 DNA (see also Table I). Thus, ϕ29 DNA polymerase residues Thr15 and Asn62 seem to be specialized in the stabilization of an editing complex, not having a role in the strand displacement capacity of the viral enzyme. In agreement with our data, it has recently been reported that substitution of the corresponding Asn residue of PRD1 DNA polymerase did not affect the strand displacement capacity of this enzyme (Zhu and Ito, 1994). Taking into account all these data, a dual role in 3′-5′ exonuclease and strand displacement appears to be restricted to residues directly acting as metal ligands, such as the Asp and Glu residues of the Exo I motif (**DxE**), the Asp residue of the Exo II motif (Nx_{2-3}**F/YD**) and the Asp residue of the Exo III motif (Yx_3**D**), or likely affecting the metal binding network, such as the Tyr residue of the Exo III motif (**Y**x_3D). To define the extent of overlap of these two functions—proofreading and strand displacement—further mutational analysis of the N-terminal domain of ϕ29 DNA polymerase will be required, selecting as targets not only residues generally conserved in proofreading DNA polymerases, but also those residues that are specific for protein-primed DNA polymerases.

Evolutionary implications

In addition to the residues directly involved in metal binding and catalysis at the 3′-5′ exonuclease active site, other residues appear to be structurally and functionally conserved at the exonuclease domain of most prokaryotic and eukaryotic DNA-dependent DNA polymerases. As shown here, the functional importance of residues Thr15 and Asn62 of ϕ29 DNA polymerase in ssDNA binding at the 3′-5′ exonuclease active site, a role that could only be anticipated from structural analysis of Pol IK complexed with DNA, completely agrees with the evolutionary conservation of these two residues. Moreover, their major importance for exonucleolysis under polymerization conditions suggests that these two residues provide important contact sites for stabilizing the primer-terminus at the 3′-5′ exonuclease active site. It can be speculated that such a functional (and structural) preservation was mainly imposed by the evolution of a proofreading mechanism in which the coordination of synthetic and degradative functions must favor immediate and selective exonucleolysis of polymerization errors.

Materials and methods

Nucleotides and proteins

Unlabeled nucleotides were purchased from Pharmacia P-L Biochemicals. [α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International plc. Restriction endonucleases were from New England Biolabs. T4 polynucleotide kinase and Pol IK were from Boehringer Mannheim. ϕ29 TP was purified as described previously (Zaballos *et al.*, 1989). The wild-type ϕ29 DNA polymerase was purified from *E.coli* NF2690 cells harboring plasmid pJLw2, as described previously (Lázaro *et al.*, 1995). ϕ29 DNA polymerase site-directed mutants T15I, N62H and N62D (this work) and D12A/D66A (Bernad *et al.*, 1989) were purified from *E.coli* BL21(DE3) pLysS cells (Studier and Moffatt, 1986) harboring the corresponding recombinant plasmids, essentially as described for the wild-type ϕ29 DNA polymerase.

DNA templates and substrates

Oligonucleotides sp1 (5′GATCACAGTGAGTAC) and sp1p (5′GATCACAGTGAGTAG), differing in the 3′ terminal base, oligonucleotide sp1c+6 (5′TCTATTGTACTCACTGTGATC), that has a 5′-extension of six nucleotides in addition to the sequence complementary to sp1, and a 4mer oligonucleotide (5′ATCA), were prepared with a DNA synthesizer from Applied Biosystems. Oligo(dT)$_{16}$ was from Pharmacia Biotech Inc. The oligonucleotides sp1, sp1p, oligo(dT)$_{16}$ and 4mer were 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. With the exception of the 4mer oligonucleotide, the rest were further purified by electrophoresis on 8 M urea–20% polyacrylamide gels. Labeled oligo(dT)$_{16}$ and 4mer were used as substrates for 3′-5′ exonucleolysis on ssDNA. Labeled oligo(dT)$_{16}$ and sp1 were used as substrates for gel retardation of ssDNA. To analyze the 3′-5′ exonuclease activity on a template/primer structure in the absence of dNTPs or under polymerization conditions, either 5′-labeled sp1 (matched 3′-terminus) or 5′-labeled sp1p (mismatched 3′-terminus) were hybridized to sp1c+6 in the presence of 0.2 M NaCl and 60 mM Tris–HCl (pH 7.5). ϕ29 DNA was obtained by proteinase K treatment of phage particles in the presence of SDS (Inciarte *et al.*, 1976), phenol extraction and ethanol precipitation. ϕ29 DNA was digested with *Eco*RI to generate fragments with 3′ recessive ends, suitable as templates for DNA polymerase activity (filling-in reaction). M13mp8 ssDNA was hybridized to the universal primer as described above, and the resulting molecule was used as a primer/template suitable to analyze processive DNA polymerization coupled to strand displacement by ϕ29 DNA polymerase. Terminal protein-containing ϕ29 DNA (ϕ29 DNA-TP) was obtained as described previously (Peñalva and Salas, 1982).

Site-directed mutagenesis and expression of ϕ29 DNA polymerase mutants

The wild-type ϕ29 DNA polymerase gene, cloned into M13mp19 (M13mp19w21; Bernad *et al.*, 1989), was used for site-directed mutagenesis, carried out essentially as described previously (Nakamaye and Eckstein, 1986), using the oligonucleotide-directed *in vitro* mutagenesis kit from Amersham International plc. The fragments carrying the different mutations were subcloned in plasmid pT7-4w2 (Lázaro *et al.*, 1995), which expresses ϕ29 DNA polymerase under the control of the T7 RNA polymerase-specific ϕ10 promoter (Tabor and Richardson, 1985). The presence of the desired mutation was confirmed by complete sequencing each ϕ29 DNA polymerase mutant gene. Sequencing was carried out by the chain termination method, using Sequenase version 2.0 from US Biochemical Corp., and a set of synthetic oligonucleotides complementary to the ϕ29 DNA polymerase gene as sequencing primers. Expression of the mutant proteins was carried out in *E.coli* strain BL21(DE3) pLysS, which contains the T7 RNA polymerase gene under the control of the IPTG-inducible *lac*UV5 promoter, and a plasmid constitutively expressing T7 lysozyme (Studier and Moffat, 1986; Studier, 1991).

3′-5′ Exonuclease assay on ssDNA

The incubation mixture contained, in 12.5 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin and the indicated amount of either wild-type or mutant ϕ29 DNA polymerase. As ssDNA substrate, either 5′-labeled oligo(dT)$_{16}$ (0.075 ng) or a 5′-labeled 4mer (0.075 ng) was used. The amount of DNA polymerase added was adjusted to obtain linear conditions (5 ng with the oligo(dT)$_{16}$ and 25 ng with the 4mer substrate). Samples were incubated at 25°C for the indicated times and quenched by adding 3 μl of gel loading buffer. Reactions were analyzed by electrophoresis in 20% polyacrylamide gels in the presence of 8 M urea, and by densitometry of the autoradiographs. Total degradation was obtained by calculating the number of catalytic events giving rise to each degradation product. From these data, the catalytic efficiency of each mutant derivative (indicated in Table I) was calculated relative to wild-type ϕ29 DNA polymerase.

3′-5′ Exonuclease assay on matched and mismatched primer-terminus

The reaction mixture was as described above, but using 0.15 ng of either the hybrid molecule spl/splc+6 (matched) or splp/splc+6 (mismatched), and 20 ng of either wild-type or mutant ϕ29 DNA polymerase. Samples were incubated at 30°C for the indicated times and quenched by adding 3 μl of gel loading buffer. Each reaction was analyzed and quantitated as described when ssDNA was used as substrate for 3′-5′ exonuclease.

ssDNA gel-retardation assay

5′-Labeled oligo(dT)$_{16}$ was used as substrate to analyze the interaction of either the wild-type or mutant ϕ29 DNA polymerase with ssDNA. The incubation mixture, in a final volume of 20 μl, contained 12 mM Tris–HCl (pH 7.5), 1 mM EDTA, 20 mM ammonium sulfate, 0.1 mg/ml bovine serum albumin, 0.075 ng of oligo(dT)$_{16}$ and the indicated amount of either wild-type or mutant ϕ29 DNA polymerase. When indicated, 5′-labeled spl was used as ssDNA substrate instead of oligo(dT)$_{16}$. After incubation for 5 min at 4°C, the samples were subjected to electrophoresis in 4% (w/v) polyacrylamide gels (80:1, monomer:bis), containing 12 mM Tris–acetate (pH 7.5) and 1 mM EDTA, and run at 4°C in the same buffer at 8 V/cm, essentially as described previously (Carthew *et al.*, 1985). After autoradiography, ϕ29 DNA polymerase–ssDNA complexes were detected as a mobility shift (retardation) in the migrating position of the labeled DNA.

Polymerase/exonuclease coupled assays

Pol/Exo balance. The hybrid molecule spl/splc+6 contains a 6-nucleotide long 5′-protruding end and, therefore, the primer strand can be used both as substrate for the 3′-5′ exonuclease activity and also for DNA-dependent DNA polymerization. The incubation mixture contained, in 12.5 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.15 ng of 5′-labeled spl/splc+6, 20 ng of either wild-type or mutant ϕ29 DNA polymerase, and the indicated concentration of the four dNTPs. After incubation for 5 min at 30°C, the reaction was stopped by adding EDTA up to 10 mM. Samples were analyzed by 8 M urea–20% PAGE and autoradiography. Polymerization or 3′-5′ exonuclease activity are detected as an increase or decrease, respectively, in the size (15mer) of the 5′-labeled spl primer.

Filling-in assay. The incubation mixture contained, in 25 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.1 mM [α-^{32}P]dATP (1 μCi), 0.2 mM dGTP and dTTP, 0.2 μg of *Eco*RI-digested ϕ29 DNA as template, and 25 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for 5 min at 30°C (conditions shown to be linear with time and amount of enzyme), the reaction was stopped by adding 10 mM EDTA–0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume, corresponding to the labeled DNA, was counted (Cerenkov radiation) and analyzed by agarose gel electrophoresis and autoradiography. To measure the 3′-5′ exonucleolytic activity coupled to polymerization, samples withdrawn immediately after incubation were also analyzed by TLC (Polygram Cel 300 PEI/UV$_{254}$) and further autoradiography. The chromatogram was developed with 0.15 M lithium formate (pH 3.0), conditions in which the 5′-dAMP migrates, whereas the DNA substrate remains at the origin. Densitometry of the spot corresponding to 5′-dAMP allowed a relative value (dAMP release relative to the wild-type) of the 3′-5′ exonuclease activity to be obtained.

DNA replication misincorporation assay

Conditions were essentially as described above for the polymerization/exonuclease coupled assay on spl/splc+6, but in this case increasing concentrations of only dATP, complementary to template positions 1, 2, 4 and 6, were added. To prevent exonucleolytic degradation of the primer-terminus, 25 μM dCTP was added. After incubation for 5 min at 30°C, samples were analyzed by 8 M urea–20% PAGE. After autoradiography, misinsertion of dAMP at non-complementary positions is observed as the appearance of extension products of the 5′-labeled spl primer (15mer) larger than the correct 17mer extension product. The misincorporation produced in each case, expressed as the ratio 18mer + 19mer /17mer + 18mer + 19mer, was determined by densitometry of the autoradiographs.

TP-primed initiation assay

The incubation mixture contained, in 25 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 0.2 μM [α-^{32}P]dATP (2.5 μCi), 0.5 μg of ϕ29 DNA-TP, 125 ng of purified TP and the indicated amount of either wild-type or mutant ϕ29 DNA polymerase. After incubation for 5 min at 30°C, the reaction was stopped by adding 10 mM EDTA and 0.1% SDS. The samples were then filtered through Sephadex G-50 spin columns, and further analyzed by SDS–PAGE as described previously (Peñalva and Salas, 1982). Quantitation was by densitometric analysis of the band corresponding to the TP–dAMP complex, detected by autoradiography.

Strand-displacement assays

Replication of primed M13 DNA. The incubation mixture contained, in 25 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (2.5 μCi), 0.25 μg of primed M13mp8 ssDNA, and 100 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 30°C, the reaction was stopped by adding 10 mM EDTA–0.1% SDS, and the samples were filtered through Sephadex G-50 spin columns. Relative activity was calculated by counting the Cerenkov radiation corresponding to the excluded volume. For size analysis, the labeled DNA was denatured by treatment with 0.7 M NaOH and subjected to electrophoresis in alkaline 0.7% agarose gels, as described previously (McDonnell *et al.*, 1977). After electrophoresis, the position of unit-length M13mp8 DNA was detected by ethidium bromide staining and then the gels were dried and autoradiographed.

Replication assay (protein-primed initiation plus elongation) with ϕ29 DNA-TP as template. The incubation mixture contained, in 25 μl, 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM ammonium sulfate, 1 mM dithiothreitol, 4% glycerol, 0.1 mg/ml bovine serum albumin, 20 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (1 μCi), 0.5 μg of ϕ29 DNA-TP, 125 ng of purified TP and 10 ng of either wild-type or mutant ϕ29 DNA polymerase. After incubation for the indicated times at 30°C, the samples were processed and the synthesized DNA was quantitated and analyzed as described above for the M13 DNA replication assay. After electrophoresis, the position of unit-length ϕ29 DNA (19 285 bases) was detected by ethidium bromide staining, and the gels were then dried and autoradiographed.

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